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Ca^{2+} signalling in the control of motility and guidance in mammalian sperm

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1. ABSTRACT

Ca^{2+} signalling in the sperm plays a key role in the regulation of events preceding fertilisation. Control of motility, including hyperactivation and chemotaxis, is particularly dependent upon $[Ca^{2+}]_i$ signalling in the principal piece of the flagellum and the midpiece. Here we briefly review the processes that contribute to regulation of $[Ca^{2+}]_i$ in mammalian sperm and then examine two areas: (i) the regulation of hyperactivation by $[Ca^{2+}]_i$ and the pivotal roles played by CatSpers (sperm-specific, Ca^{2+}-permeable membrane channels) and intracellular Ca^{2+} stores in this process and (ii) the elevation of $[Ca^{2+}]_i$ and consequent modulation of motility caused by progesterone including the ability of progesterone at micromolar concentrations to cause sperm hyperactivation and/or accumulation and the recent discovery that progesterone, at picomolar concentrations, acts as a chemoattractant for mammalian sperm.

2. INTRODUCTION

The early stages of sperm-egg interaction depend heavily upon regulation of the activities of the male gamete. Failure of a sperm to respond appropriately to cues from the oocyte and (in mammals) female tract will render it unable to fertilise. Ca^{2+} signalling is pivotal to these processes (1, 2). It has been known for more than 30 years that the acrosome reaction, an event which is not only a requirement for fertilisation, but must occur at the appropriate place and time, is dependent (in mammalian and non-mammalian cells) on Ca^{2+} (3, 4). It is now believed that this process requires a combination of Ca^{2+} influx from the extracellular environment and mobilisation of stored Ca^{2+} (5-7). Much less clearly understood but also highly important is the role of Ca^{2+}-signalling in capacitation (8). However, for the last few years the area of most intense interest and activity has been the role(s) of Ca^{2+}-signalling in the regulation of sperm 'behaviour',

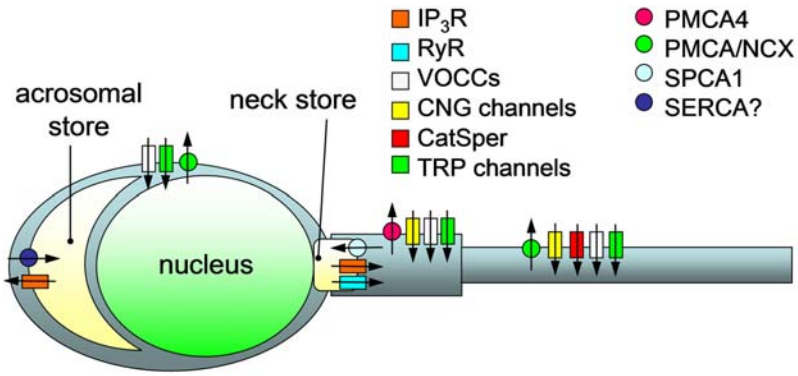

Figure 1. Regulation of $[Ca^{2+}]_i$ in mammalian sperm – components of the toolkit. The diagram illustrates the classes and putative locations of pumps, channels and intracellular storage organelles that are believed to be present in mammalian sperm and contribute to $[Ca^{2+}]_i$ regulation. Rectangles indicate channels, circles indicate pumps. Normal direction of Ca^{2+} movement is shown by arrows. Locations are approximate, indicating presence on the sperm head, midpiece and principal piece or on intracellular organelles. Where a class of pump or channel is shown in more than one location this reflects reported distribution of all members of that class and immunolocalisation may have shown restriction of specific gene products to only one location. Question mark against SERCAs indicates that the presence or functional significance has been disputed (see text). For clarity, mitochondria in the sperm midpiece, which accumulate Ca^{2+} into the matrix space via a uniporter on the inner membrane (driven indirectly by e^- transport) and contribute significantly to Ca^{2+}-buffering, are not shown.

changes in the characteristics and directional component of motility including activation, hyperactivation and chemotaxis (9-12). In this review we briefly survey current ideas about the sperm Ca^{2+}-signalling 'toolkit': how sperm regulate $[Ca^{2+}]_i$ and generate appropriate Ca^{2+} signals in response to stimuli and cues from their environment. Then we examine what is known about the participation of the Ca^{2+} toolkit and Ca^{2+} signals in regulating two important 'behaviours' of mammalian sperm – hyperactivation and the response to progesterone. Current ideas on the roles of Ca^{2+}signalling in chemotactic responses of sea urchin sperm exposed to sperm activating peptides and mammalian sperm responding to olfactory stimulants are covered in the contributions by B Kaupp and M Spehr.

3. $[Ca^{2+}]_i$ REGULATION IN MAMMALIAN SPERM: THE SPERM'S Ca^{2+}-SIGNALLING TOOLKIT

3.1. Ca^{2+} handling at the plasma membrane – Ca^{2+} channels and pumps

The importance of Ca^{2+} channels in the sperm plasma membrane, and their distribution and regulation, is well established (13, 14; Figure 1a). Similarly to Ca^{2+}, cAMP is of central importance in sperm function and it is therefore not surprising that cyclic nucleotide-gated (CNG) channels have been detected in mammalian sperm. Weyand *et al* (15) cloned a novel Ca^{2+}-permeable CNG channel (of the type found in vertebrate photoreceptors and olfactory neurons) from bovine testis that was expressed in sperm. Subsequently the same group showed that CNG channels were restricted to the flagellum (the beta subunit being present only in the principal piece). These observations are particularly exciting in the light of two recent observations: i) activation of olfactory receptors, apparently linked to adenylate cyclase, leads to Ca^{2+} influx and consequent chemotactic behaviour in human sperm and; ii) K^+-selective CNG channels cause a hyperpolarisation of the membrane that induces a chemotactic turn in sea urchin sperm (see contributions by B. Kaupp and M. Spehr). Evidence consistent with the presence of functional CNG channels in intact sperm has been reported. Liberation of cGMP caused elevation of $[Ca^{2+}]$ in the flagellum (16). Manipulation of cGMP or cAMP levels in mouse spermatozoa induced a transient elevation of $[Ca^{2+}]_i$ lasting 20–60 s (17). This effect was greatly reduced in low-Ca^{2+} saline or in the presence of Ca^{2+}-channel blockers, suggesting that Ca^{2+} was mobilized through cyclic nucleotide-regulated channels, cGMP being significantly more effective in elevating $[Ca^{2+}]_i$. However, the significance of CNG channels mammalian in sperm function is still far from clear, since no impairment of reproductive function has been shown in any of the mouse CNG channel subunit-null models (18). More specifically, treatment of mouse sperm with bicarbonate, which elevates [cAMP] by activation of soluble adenylate cyclase, does not induce significant Ca^{2+}-influx unless the stimulus is paired with alkalinisation (10). Furthermore, some of the observed effects of cyclic nucleotides may reflect actions on CatSpers (see below), since elevation of $[Ca^{2+}]_i$ induced by treatment with membrane-permeant cAMP or cGMP is lost in sperm of CatSper-null mice (19).

Over the past 15 years many laboratories have shown evidence for the expression of voltage-operated Ca^{2+} channels (VOCCs) in sperm (both mammalian and non-mammalian) and their participation in induction of the acrosome reaction by biological agonists. Mouse and bovine sperm exposed to solubilised zona or purified zona protein 3 (ZP3) show an increase in $[Ca^{2+}]_i$ and undergo an acrosome reaction. These responses are dependent upon $[Ca^{2+}]_o$ (indicating participation of Ca^{2+}-influx) and both the elevation of $[Ca^{2+}]_i$ and induction of acrosome reaction are subject, with similar potencies, to inhibition by organic and inorganic antagonists of VOCCs (13, 20-24). Immunostaining and *in-situ* hybridisation of sperm preparations, western blotting of sperm protein extracts and PCR of spermatid cDNA libraries all indicated the presence

of numerous VOCCs in sperm. The types which have been detected by these various techniques vary widely in their voltage-sensitivity and kinetics of activation and inactivation and are apparently restricted in their distribution such that different parts of the sperm membrane might respond differently (generating different sorts of Ca^{2+} signal) in response to changes in membrane potential (13, 25, 26). Moreover, pharmacological investigation of depolarisation-induced and agonist-induced elevation of $[Ca^{2+}]_i$ in mouse and human sperm has suggested the participation of at least two channel types in supporting Ca^{2+} influx (27, 28).

However, when electrophysiological (whole cell patch clamp) studies were carried out on mouse and human spermatogenic cells (spermatocytes and spermatids) only transient (T-type) currents were observed upon application of depolarising pulses (23, 29-31), though this current may include two, pharmacologically separable components (28). More recently the presence of the cytoplasmic droplet in immature (epididymal) mouse sperm has been exploited to apply whole cell patch clamp to these cells. Again only low-voltage activated, transient (T-type) currents were seen (32). Application of cell-attached (single channel) patch clamp recording to map the distribution of functional ion channels in the head of human sperm revealed a number of channel types which were clearly differentially distributed, but provided no evidence for the presence of active VOCCs (33).

This discrepancy between the apparent richness of VOCC expression detected by molecular approaches, and the paucity of currents detected in intact cells is yet to be resolved. The high-voltage activated (non-T-type) channels detected by molecular studies may be 'dormant', becoming available for activation only upon modulation occurring during capacitation or perhaps following activation of membrane receptors by agonists encountered in the female tract. Alternatively, the sensitivity of molecular techniques may have detected 'vestigial' channel proteins which were active in the developing male germ cell but are no longer functional.

Transient receptor potential (TRP) channels are a family of Na^+ and Ca^{2+}-permeable channels that resemble structurally the superfamily of voltage-gated channels. These channels vary greatly in their mode of activation and are believed to participate in a wide range of processes involving influx of Ca^{2+}, including mechanosensitivity, sensing of pain and thermosensitivity (34, 35). These channels have been detected in mouse sperm (36, 37) and TRP2 is implicated in the signal cascade that causes acrosome reaction in zona-bound sperm (36). However, several TRPs have been detected in mouse sperm flagellum (37) and TRPC1,3,4 and 6 have been localised to the flagellum of human sperm (38). SKF96365, a blocker of TRPC channels, significantly inhibited human sperm motility (38).

Recently, a novel Ca^{2+} -selective sperm-specific channel has been described (39). These 'CatSpers' are unique to sperm, being localised to the principal piece of the flagellum (19, 40, 41). There are 4 CatSper genes (CatSper 1-4), all coding for proteins structurally similar to subunits of voltage-operated channels. The Catsper proteins are believed to form (probably heteromeric) tetramers and knockout of any one of the four genes is sufficient to prevent functional expression of CatSper channels, leading to effects on motility and fertility (42, 43; see below). To date most work has been done on mouse CatSpers, where work with CatSper null mice has shown the channel to be extremely important in regulation of motility, particularly hyperactivation (see below). However, molecular studies have shown that CatSpers are expressed in human testis (19, 40, 44) and CatSper mutations may be significant in some forms of human male infertility (45, 46).

All cells use ATP to extrude Ca^{2+} at the plasmalemma; either directly, by plasma membrane Ca^{2+}-ATPases (PMCAs), or indirectly by Na^+-Ca^{2+} exchangers (NCXs). There are four isoforms of the PMCA (PMCA1–4), PMCA1 and PMCA4 being most common (47). PMCA (primarily PMCA4) protein has been detected in rat spermatids (48) and mouse spermatozoa (49, 50). PMCA4 is located primarily in the principal piece of the sperm flagellum (49-51). In PMCA4 null mice the ability to hyperactivate is lost, possibly due to inability to regulate $[Ca^{2+}]_i$ under hyperactivating conditions (50); see below). The NCX exports one Ca^{2+} in exchange for influx of three Na^+, using energy derived from the Na^+ gradient at the cell membrane, and thus indirectly from activity of the Na^+, K^+-ATPase (52). Using RT–PCR, two splice variants from the NCX1 gene (NCX1.3 and NCX1.7) were found in rat testis (53) and mRNA for the NCKX3 isoform was detected in mouse testis (54). Pharmacological evidence indicates that that activity of NCX is crucial for maintenance of $[Ca^{2+}]_i$ and motility in human sperm (55, 56). Immunostaining localised NCX primarily to the acrosomal area and the midpiece (56). NCX expressed in the tail of sea urchin sperm plays a similarily important role in regulating $[Ca^{2+}]_i$, such that blockade of the exchanger increases $[Ca^{2+}]_i$ and impairs motility (57).

3.2. Intracellular Ca^{2+} handling - Ca^{2+} stores

Only in the last 5-10 years has it become apparent that mammalian (including human) sperm possess intracellular Ca^{2+} stores (Figure 1). Work from several groups has shown that the acrosome functions as a store in a manner analogous to the ER of somatic cells, emptying upon IP_3R activation (7, 58-60). IP_3 receptors are localised to the anterior sperm head and are lost upon acrosome reaction consistent with localisation to the outer acrosomal membrane (59). Ca^{2+} storage by the acrosome has recently been shown in human and mouse spermatozoa and this stored Ca^{2+} can be released by IP_3 and Ca^{2+} ATPase inhibitors (5, 7, 60). It has been suggested that 'the mouse sperm acrosome is a Ca^{2+} store that regulates its own exocytosis through an IP_3 Ca^{2+} mobilization pathway' (7). Similar conclusions have been drawn for human spermatozoa (60). The role of this store in the mature sperm prior to acrosome reaction is unknown.

Intriguingly, it now appears that there is at least one other Ca^{2+} store, in the neck/midpiece region of

mammalian sperm (61-64; Figure 1). Ho and Suarez (61, 62) have described a Ca^{2+} store in the neck region of mouse sperm. They suggest that the redundant nuclear envelope, which extends from the back of the nucleus, acts as a releasable store that regulates activity of the flagellum (see below). IP_3 receptors have been localised to this region. In human sperm it has been shown that a store, apparently situated in the neck region, can be mobilised independently of IP_3 signalling, apparently dependent upon activation of ryanodine receptors (RyRs) (65). It is possible that storage in this area of mammalian sperm involves more than one compartment. Both the redundant nuclear envelope (excess nuclear envelope 'released' during nuclear condensation) (61) and calreticulin-containing vesicles in the cytoplasmic droplet have been proposed (63). The mitochondria of sperm also contribute to Ca^{2+} buffering (49). The possibility that Ca^{2+} signals in the midpiece of human sperm merely reflect a normal Ca^{2+} release and (e^--transport-dependent-) re-accumulation by mitochondria has been addressed by using high doses of uncouplers (2,4 dinitrophenol and carbonyl cyanide m-chlorophenylhydrazone). Ca^{2+} mobilisation and accumulation were not affected (65, Machado-Oliveira, unpublished data). However, SPCA has been localised to the giant mitochondrion in sea urchin sperm (66) and is expressed throughout the mitochondrial midpiece of human cells, not just in the anterior region where RyRs are normally detected. Thus this store might comprise two components, a small, anteriorly-placed 'trigger' expressing RyRs and a larger mitochondrial component that can accumulate Ca^{2+} by ATPase activity. Regardless of its identity, the position of this Ca^{2+} source, and the evidence of physiological studies suggest that it plays a key role(s) in regulating flagellar activity during events leading up to fertilisation.

4. HYPERACTIVATION

Hyperactivation of mammalian sperm is a change in flagellar activity from activated (a high frequency, low-amplitude beat that causes progressive movement in low-viscosity, aqueous media) to a much more vigorous 'hyperactivated' pattern. Though the characteristics of hyperactivated motility are difficult to define, since they apparently vary somewhat between species, typically the flagellum forms deeper bends, the beat becomes asymmetric and the frequency of beating is significantly reduced (67-69). When cells are suspended in low viscosity medium this results in marked lateral movements of the sperm head and often a circular swimming pattern or a non-progressive, whiplash-like, tumbling movement. This change in motility normally occurs in the female tract, simultaneously with a suite of biochemical and structural changes, that render the sperm capable of fertilisation (collectively referred to as capacitation; 8) and can be considered as part of this process. However, use of enzyme inhibitors and physiological manipulations suggest that hyperactivation is regulated by different signalling pathways to other aspects of capacitation and can occur separately (70). It is likely that hyperactivation plays a number of roles during progress of the sperm to the oocyte. Progressive movement in viscous and visco-elastic media is significantly improved when sperm are hyperactivated (71) such that penetration into and within mucus, and also penetration of the cumulus surrounding the oocyte will be improved in hyperactivated cells. Importantly, penetration of the zona is greatly increased in hyperactivated cells (72) and zona penetration may even be impossible without hyperactivation (73). Any or all of these effects of hyperactivation may be crucial.

4.1. Ca^{2+} in the regulation of hyperactivation

Both Ca^{2+} signalling and cAMP-mediated signals are believed to participate in the induction and regulation of hyperactivation. Treatment of sperm to elevate $[Ca^{2+}]_i$ (ionophore A23187) can reversibly induce hyperactivation (74) and $[Ca^{2+}]_i$ is clearly elevated in hyperactivated sperm (9). Significantly, this increase in $[Ca^{2+}]_i$ is particularly marked in the flagellum (75). However, exposure of mammalian sperm to membrane-permeant phosphodieterase inhibitors (a manipulation intended to reset the balance of cAMP synthesis/degradation, thus elevating [cAMP]) also causes increased levels of hyperactivated motility (76-78). Thus either or possibly both of these second messengers regulate the transition from activated to hyperactivated motility. Attempts to determine, more precisely, the regulation of this process have employed detergent extracted (de-membranated) sperm 'models'. When these sperm models are reactivated in medium containing Mg-ATP and EGTA (1-100 nM $[Ca^{2+}]$ depending on species) flagellar beating restarts and is typically rapid with low amplitude symmetrical bends (79-81). Elevation of $[Ca^{2+}]$ in the medium (and thus 'cytoplasmic' $[Ca^{2+}]$ in the sperm 'model') causes an increase in flagellar curvature of the principal bend and, consequently, increased beat asymmetry, resulting in hyperactivated type motility (80). When $[Ca^{2+}]$ is raised to very high levels (500 microM–1 mM) rodent, human and bovine de-membranated sperm 'models' can become immotile, with a strongly curved flagellum (79, 80, 82). In contrast, though addition of cAMP to de-membranated mammalian sperm seems to enhance flagellar reactivation (83), it apparently has very little effect on beat mode (79, 80, 84). In a recent study on de-membranated macacque sperm, Ishijima *et al* (81) concluded that $[Ca^{2+}]$ caused a concentration-dependent increase in asymmetry without significant effects on beat frequency or wavelength. Asymmetry was largely due to changes in the behaviour of the sperm midpiece. When cAMP (50-150 microM) was included with stimulating levels of Ca^{2+} the hyperactivated beat became more symmetrical and beat frequency was reduced.

4.2. CatSpers and hyperactivation

It appears that the primary determinant of the change to hyperactivated motility is increased cytoplasmic $[Ca^{2+}]$. How is Ca^{2+} mobilised into the cytoplasm to induce hyperactivation and how is it regulated so that the Ca^{2+} signal is directed to the right part of the cell at the right time? Recently a series of important studies have identified and characterised both membrane Ca^{2+} channels and intracellular Ca^{2+} stores that are apparently pivotal to this process. The CatSpers (see section 3.1 above) play a particularly important role in this respect. CatSpers were

first described in 2001 (19, 40) as sperm-specific ion channel subunits with a structure resembling that of voltage-gated cation channels. Male mice null for CatSper1 were shown to be infertile, their sperm having impaired motility and lacking a cAMP-induced Ca^{2+} influx that was present in wild type cells. Further investigation clarified the Catsper motility phenotype, showing that, in sperm lacking functional CatSper channels (CatSper 1 or CatSper 2 null), activated (progressive) motility was near normal but the cells failed to hyperactivate when incubated under conditions that normally induce capacitation (and hyperactivated motility) (85). Patterns of protein tyrosine phosphorylation and ability to undergo acrosome reaction when stimulated with zona pellucida showed that the cells were indeed capacitated (10, 73), indicating that the lesion was a specific failure of hyperactivation. Male mice with this defect were completely infertile and the sperm failed to fertilise any oocytes *in vitro* (73). However, removal of the zona pellucida resulted in normal rates of *in vitro* fertilisation, indicating that infertility was a direct result of failure of the sperm to penetrate the zona due to their failure to hyperactivate (73).

Analysis of the physiology of these CatSper-null cells shows that, though they are in many respects normal, they fail to generate a rapid increase in $[Ca^{2+}]_i$ when they are exposed to depolarising conditions in the presence of 15 mM bicarbonate, whereas in control cells a robust increase occurs (10, 49). This increase in $[Ca^{2+}]_i$ is rapid (half time ≈60s), dependent on the presence of Ca^{2+} in the medium and appears to involve generation of cAMP by the bicarbonate-sensitive, soluble adenylate cyclase (and consequent activation of PKA; (49). Interestingly, treatment with 15 mM bicarbonate causes a Ca^{2+}-dependent increase in flagellar beat rate and reduction of flagellar asymmetry which is also dependent on cAMP and PKA, but which is not lost in cells lacking CatSper channels (10). Kirichok *et al* (41) have successfully applied whole-cell patch clamp to epididymal mouse sperm by using the cytoplasmic droplet of these cells to obtain gigaseals and membrane 'breakthrough'. These authors detected a Ca^{2+} current ($I_{CatSper}$) that was absent in CatSper-null cells. The current was weakly voltage sensitive, but this sensitivity showed a pronounced negative shift (to physiological values of membrane potential) when pHi was raised from it basal value of <7 to ≈7.5 or 8 (41). Recently the same laboratory has detected a K^+ current (KSper) in murine sperm, also localised to the principal piece of the sperm. This current activates in response to intracellular alkalinisation, leading to hyperpolarisation and an increased driving force for Ca^{2+} entry through CatSper channels (86).

Studies on human sperm have shown that elevation of pH_i (intracellular alkalinisation using NH_4Cl) causes a dose-dependent increase in $[Ca^{2+}]_i$ and also enhances depolarisation-induced Ca^{2+} influx (87), effects which may relate to expression of CatSpers in human sperm. This response to intracellular alkalinisation is apparently enhanced by capacitation (87, 88). In bovine sperm exposure to NH_4Cl also raises $[Ca^{2+}]_i$, an effect that has a longer latency than the NH_4Cl-induced rise in pH, consistent with a causative link between these two responses in which elevation of pH is required for mobilisation of Ca^{2+} (70). Hyperactivation accompanied the NH_4Cl-induced rise in $[Ca^{2+}]_i$. Both this hyperactivation and the increase in $[Ca^{2+}]_i$ were sensitive to removal of Ca^{2+} from the extracellular medium, though this did not reduce the alkalinising effect of NH_4Cl (70). Interestingly, in the whole cell patch clamp experiments carried out by Kirichok *et al* (41) cyclic nucleotides did not potentiate $I_{CatSper}$, suggesting that 'the (previously) observed CatSper1-dependent rise in $[Ca^{2+}]_i$ induced by cyclic nucleotides did not result from direct modulation of the CatSper channel' (41). This is consistent with the requirement for PKA activation in the enhancement of voltage-induced Ca^{2+} influx caused by bicarbonate, but how activation of PKA regulates the channel (through alkalinisation or though another more direct mechanism) is not yet clear.

4.3. Ca^{2+} stores and hyperactivation

Another potentially important regulator of Ca^{2+} in the control of hyperactivated motility is the liberation of Ca^{2+} from intracellular stores. Suarez and colleagues reported that the redundant nuclear envelope may act as a Ca^{2+} store (61). IP_3 receptors (IP_3Rs) and calreticulin were localised, at histological and electron microscope levels, to these intracellular membranes (61, 62). Treatment with thimerosal or high concentrations of thapsigargin elevated Ca^{2+} in the neck region in bovine sperm, in the absence of extracellular Ca^{2+} (indicating that Ca^{2+} was being mobilised from intracellular stores) and induced hyperactivation (61, 62). Mouse sperm were similarly able to undergo hyperactivation by mobilisation of stored Ca^{2+} when flux at the membrane had been abolished by extracellular Ca^{2+}-buffering, though Ca^{2+} influx across the membrane is probably necessary to maintain this type of motility for a prolonged period (89). When sperm from CatSper 1 and CatSper 2 null mice were stimulated in this way they responded similarly, despite the absence of CatSper channels, confirming that mobilisation of stored Ca^{2+} in the neck region is capable of supporting hyperactivation independently of influx through CatSpers (89). Recently it has been shown that upon activation of Ca^{2+} influx into the principal piece of the mouse sperm flagellum, (evoked by cell-permeant cyclic nucleotide analogues or by alkalinisation) there is not only an elevation of flagellar $[Ca^{2+}]$ (and thus hyperactivation) but also (with a delay of ≈3 s) a propagation of the signal into the midpiece and sperm head. $[Ca^{2+}]_i$ elevation in the head persisted much longer than the flagellar signal, possibly reflecting relatively poor Ca^{2+} buffering in the head. Both the initial Ca^{2+} elevation and the forward propagation were lost in CatSper-null cells (90). The authors considered that this was likely to be an active process rather than passive spread by diffusion, thus it may be that activation of CatSpers recruits mobilisation of Ca^{2+} stored in the sperm neck region, possibly by Ca^{2+}-induced Ca^{2+} release (see below). Interestingly, spread of the Ca^{2+} signal into the midpiece leads to elevated NADH fluorescence suggesting that the mitochondria accumulate Ca^{2+} under these conditions, possibly having significant effects on mitochondrial metabolism (90). The occurrence of these signals under

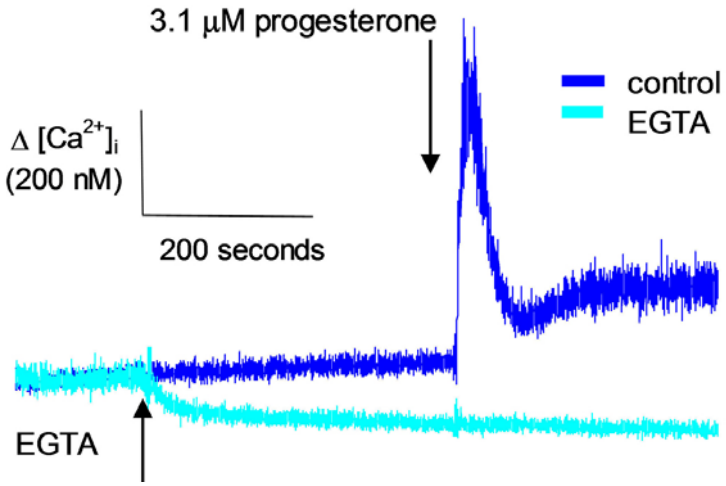

Figure 2. $[Ca^{2+}]_o$ elevation in a suspension of human sperm upon stimulation with 3 microM progesterone. A rapid increase in $[Ca^{2+}]$ occurs upon application of progesterone (arrow), followed by a plateau (blue trace). When cells are pre-incubated for 2-3 min in medium supplemented with EGTA (arrow; $[Ca^{2+}]_o$ $<5*10^{-7}$ M; pale blue trace), both components are abolished. Calibration shows delta $[Ca^{2+}]_i$ (200 nM) and time (200 seconds). Resting $[Ca^{2+}]_I$ is 120-150 nM but this decreases rapidly upon exposure to EGTA.

physiological conditions, rather than in response to artificially imposed, direct, strong activation of CatSpers, is yet to be established. However, these data suggest that mechanisms for integration of energy usage and generation might exist in mature sperm.

5. PROGESTERONE AND GUIDANCE OF HUMAN SPERM

5.1. Sensitivity of sperm to progesterone

The steroid hormone progesterone (4-pregnene-3,20-dione), which is synthesised by granulosa cells of the follicle, plays an important role in maturation of the oocyte in mammals and other vertebrates (91). After ovulation these cells still surround the oocyte (the cumulus oophorus) and continue to manufacture the hormone. Thus sperm in the mammalian female tract not only are exposed to progesterone which 'leaks' from the circulation, but must approach and penetrate a layer of progesterone secreting cells before they contact the egg. The 'classical' mode of action of steroids is to enter the cell (steroids are hydrophobic and cross the cell membrane) and interact with a cytoplasmic receptor, leading to regulation of gene transcription. However, it is now known that in many cell types steroids have effects that are exerted by mechanisms other than regulation of gene transcription (91-94). A particularly striking and well-documented example of this is the action of progesterone on human spermatozoa. Upon exposure to micromolar concentrations of progesterone, human sperm immediately (<3 s) generate a rapid increase in $[Ca^{2+}]_i$ which peaks within <15 s (at 37°C), followed by a prolonged plateau phase (95; Figure 2). This effect is far too rapid to be dependent upon gene transcription and is still observed when progesterone is conjugated to BSA (95, 96). Though use of BSA-conjugated progesterone does not absolutely preclude interaction with an intracellular receptor, there seems little doubt that the rapid action reflects a non-genomic effect. There is considerable and ongoing controversy regarding the nature of this action, which may involve 'classical' progesterone receptors (or truncated versions thereof) having non-transcriptional effects, novel membrane-associated receptors or even actions of the steroid on other membrane proteins not normally classified as receptors. Correia *et al*, (97) provides a good summary of the current situation.

5.2. Biological effects of progesterone on human sperm

The sensitivity of sperm to progesterone is well established, as is the rapid, Ca^{2+}-mobilising action of this steroid, but an understanding of the biological significance of this sensitivity has proved elusive. The best-characterised and most-studied effect of progesterone on human sperm is the induction of the acrosome reaction, an action that almost certainly underlies the acrosome reaction-inducing effect of follicular fluid (98). Stimulation of the acrosome reaction by progesterone *in vitro* is dose-dependent, with a potency which closely parallels induction of $[Ca^{2+}]_i$ elevation (95, 99, 100). On the basis of these and many other observations, it has been suggested that, *in vivo*, progesterone acts on mammalian sperm as an inducer or co-inducer of acrosome reaction (with the zona pellucida). A problem with this model is that 'premature' acrosome reaction (before zona binding) may well compromise the sperm's fertilising ability (101). However, another possibility is that stimulation by microM-mM doses of progesterone does not, under physiological conditions, induce acrosome reaction in healthy cells, but 'primes' the cell, preparing it to respond strongly upon binding the zona, enabling exocytosis of the acrosome (102, 103). *In vivo*, the spermatozoon is likely to encounter the progesterone stimulus in increasing concentration as it approaches the cumulus-oocyte complex. When human spermatozoa are exposed to such a progesterone concentration gradient priming of ionophore-induced acrosome reaction is observed at pM concentrations of the steroid (104).

An alternative function for the sensitivity of sperm to cumulus-synthesised progesterone is a role in guidance of sperm to the oocyte. The steroid may form a gradient, at least in the immediate vicinity of the cumulus-oocyte complex, or even within it (105, 106). Several groups have investigated the chemotactic activity of follicular fluid, which typically contains between 5 and 50 microM progesterone (107), though recently levels as low as 54 nM have been reported (108). That follicular fluid acts as a chemoattractant for human spermatozoa seems beyond dispute (107, 109, 110). However, the question of whether progesterone is the (or a) chemoattractant present in follicular fluid has proved difficult to resolve. Vadillo Ortega *et al* (111), Silwa (112), Villanueva-Diaz *et al* (113); Wang *et al* (114) and Jeon *et al* (110) all reported detecting chemotactic activity of progesterone at micromolar concentrations. In contrast Jaiswal *et al* (109) reported that the chemotactic activity of follicular fluid was not affected by removal of steroids using dextran-coated charcoal, though the ability of follicular fluid to induce hyperactivation was lost. They further reported that progesterone, at micromolar concentrations, was not a potent chemoattractant and did not cause turning of sperm up gradient, but induced hyperactivation in a significant proportion of cells. They concluded that the ability of progesterone to cause accumulation of sperm in chemotaxis assays might actually be because the excitatory action of the steroid caused sperm 'trapping'. Any hyperactivating effect of progesterone would reduce the progressive component of motility, such that sperm, having arrived randomly in an area of high progesterone concentration would not readily leave it (109). Most recently a study by Teves *et al* (106) has shown that at very low concentrations (1-100 pM) progesterone acts as a true chemoattractant for mammalian sperm. A one dimensional concentration gradient, generated by pM progesterone, is able to induce a chemotactic response, causing orientation of motility up-gradient in 5-10% of cells. This is believed to reflect activity of the capacitated sub-population (106). The ability of progesterone to have two quite different effects, at different concentrations, is consistent with the report of Luconi *et al* (115) that human sperm possess two receptors for progesterone. One is poorly-specific and the other (which has a higher affinity) shows specificity for progesterone over other steroids. Estimated K_ds for the two receptors (derived from analysis of displacement assays) were 600 pM and 26 microM. However, analysis of $[Ca^{2+}]_i$ responses gave EC_{50} values of 55 nM and 40 microM. It is thus not clear how these data relate to the responses to stimulation with pM and microM doses of progesterone used in chemotactic assays.

5.3. Role of $[Ca^{2+}]_i$ in the biological effects of progesterone

Due, at least in part, to the practical difficulties of obtaining human zona pellucida, progesterone is the most-studied and best-characterised Ca^{2+}-mobilising agonist of human sperm. It has been shown by many laboratories that buffering of $[Ca^{2+}]_o$ to sub-micromolar levels with EGTA abolishes progesterone-induced elevation of $[Ca^{2+}]_i$, consistent with the effect being mediated entirely by influx of Ca^{2+} through plasma membrane channels (95; Figure 2). The nature of these channels or their mechanism of activation is far from clear, but it seems unlikely that progesterone stimulation results in activation of VOCCs (87, 116-118). More recently we have shown that the response to progesterone in human (and therefore probably other mammalian) sperm is complex, involving at least two components (65, 100, 119, 120). Furthermore, when progesterone is applied as a slowly rising concentration ramp, to represent more closely the stimulus that a sperm might encounter *in vivo*, a very different response is observed. No initial transient occurs but instead there is a slow, tonic rise in $[Ca^{2+}]$ upon which, in a proportion of cells, $[Ca^{2+}]$ oscillations are superimposed (65; Figure 3). These oscillations reflect cyclic mobilisation of stored Ca^{2+} and are apparently mediated by Ca^{2+}-induced Ca^{2+} release upon activation of a ryanodine receptor or ryanodine receptor-like protein (64, 65, 120). The oscillations are resistant to the specific sarcoplasmic-endoplasmic reticulum blocker thapsigargin but are inhibited by bis-phenol at doses that inhibit secretory pathway Ca^{2+} ATPases (SPCAs; 121, 122). The nature of the store that is mobilised by stimulation with progesterone is yet to be clearly elucidated but the $[Ca^{2+}]_i$ transient, ryanodine receptors (visualised in live cells by BODIPY-ryanodine or in fixed cells by antibodies against ryanodine receptors) and secretory pathway Ca^{2+}-ATPases are all localised to the sperm neck and midpiece region (64, 65, 122). Thus the store mobilised by progesterone might well be the same as that previously identified in human sperm by the presence of calreticulin and IP_3Rs (63) and equivalent to the store that contributes to hyperactivation in bovine sperm (61, 62).

When sperm are stimulated by a progesterone gradient (such that store mobilisation occurs but in the absence of major, phasic Ca^{2+} influx, (65; Figure 3), there is no measurable induction of acrosome reaction. Instead, associated with each $[Ca^{2+}]_i$ peak there is a modulation of flagellar beat. Flagellar excursion increases (65) and in many cells there is a clear flexure of the proximal flagellum, always in the same direction (Figure 3). This might be viewed as a brief, modest activation of the processes that occur when mobilisation of stored Ca^{2+} induces hyperactivation (89). We have speculated that this effect might facilitate penetration of the zona pellucida (64). However, another possibility is that regular turns, associated with each $[Ca^{2+}]_i$ elevation, contribute to sperm trapping. Ultimately this response must be investigated in free-swimming cells or cells penetrating cumulus. Furthermore, the effects on orientation of free-swimming cells must be investigated in media of viscosity equivalent to the environment within the female tract.

All studies on chemotaxis of sperm from mammals (including humans), other vertebrates and marine invertebrates indicate that $[Ca^{2+}]_i$ signalling is pivotal to the process (see contributions by B. Kaupp and M. Spehr). Thus it is pertinent to ask whether progesterone-induced $[Ca^{2+}]_i$ signals in human sperm play a role in the recently reported chemotactic action of the steroid, or whether this might be achieved through a Ca^{2+}-independent mechanism? Initial observations indicate that buffering of extracellular Ca^{2+} and blockade of Ca^{2+}-permeable channels impair chemotactic responses, indicating that Ca^{2+} influx may be required. Intriguingly, pre-treatment with the adenylate

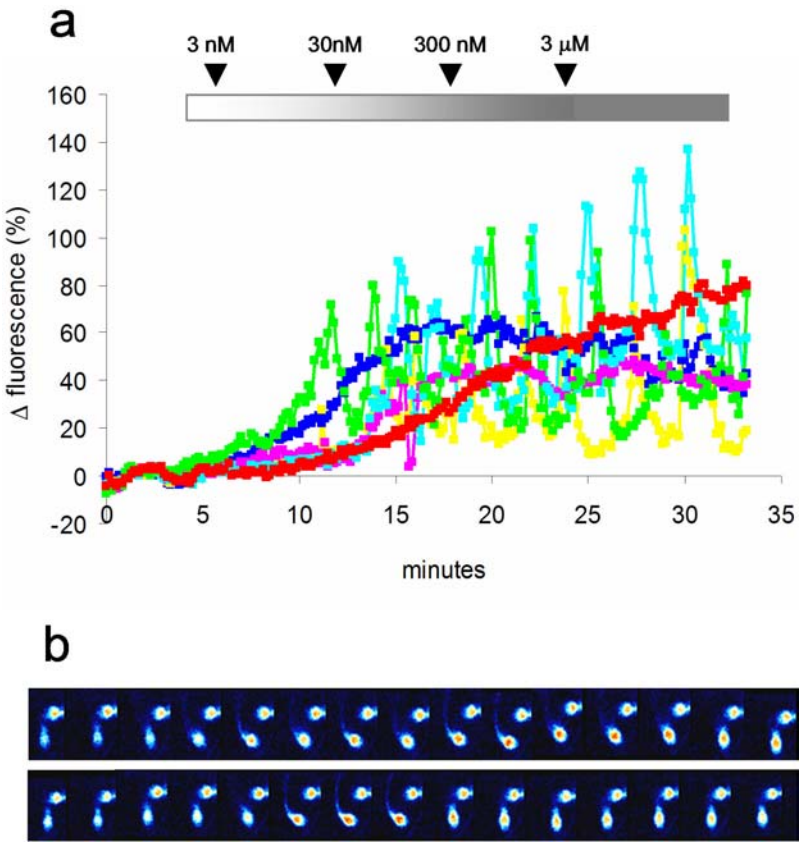

Figure 3. $[Ca^{2+}]_i$ signals induced by exposure to a rising gradient of progesterone. a: Six individual cell records, each shown by a different colour, from immobilised cells stimulated with a progesterone gradient (indicated by shaded bar above traces) such that concentration of the hormone rises from 0-3 microM over a period of approximately 20 min. Markers above the bar show estimated progesterone concentrations (from calibration of gradient maker and ELISA analysis of outflow from incubation chamber. Responses are detectable at concentrations below 3 nM. In many cells a gradual increase in $[Ca^{2+}]_i$ occurs (blue, red and pink traces) but in up to 50% of cells $[Ca^{2+}]_i$ oscillations are superimposed on this rise (yellow green and pale blue traces) which continue for the duration of recording. Axes show time in minutes and normalised (%) change in fluorescence of the Ca^{2+}-indicator Oregon Green BAPTA 1. b: Pseudocolour images showing two extracts from an image series. 'Warm' colours show high $[Ca^{2+}]_i$. Images are at 1.5s intervals and each series shows a cycle of $[Ca^{2+}]_i$ oscillation but the intervening period is not shown in full. The cell in which $[Ca^{2+}]_i$ oscillations are occurring, which is attached to the coverslip by the head and distal flagellum, clearly moves during each $[Ca^{2+}]_i$ elevation due to asymmetric bending of the proximal flagellum. Reproduced with permission from (65).

cyclase inhibitor by 2',5' dideoxyadenosine (123, 124) abolishes the chemotactic response to progesterone (125), but there is, as yet, no indication of how cAMP might act in the control of chemotactic behaviour. From the available evidence it is not possible to draw any conclusions about the role of Ca^{2+}, but two points may be considered. Firstly, do sperm mobilise Ca^{2+} in response to pM doses of progesterone? Previous work on progesterone-induced Ca^{2+}-signalling in mammalian sperm has used concentrations of nM-microM, primarily on the basis that follicular fluid and the cumulus matrix appear to contain progesterone at microM or near-microM concentrations. However, a number of studies have addressed the dose-dependence of progesterone-induced Ca^{2+} signalling. Luconi *et al* (115) reported a threshold of approximately 10 nM, Blackmore *et al*, (126), Schaefer *et al*, (127) and ourselves (100) observed a threshold at or just below 1 nM. However, all of these assays were carried out using a fluorimetric approach in which the detected signal is averaged across the full cytoplasmic volume of the entire population. Thus, if only 5-10% of cells respond, and if the response occurs in only a proportion of the cytoplasmic volume, these estimates of threshold may be considerably exaggerated. In this respect it is of interest that in cells exposed to 100 pM progesterone (applied as a step stimulus) a small sustained rise in $[Ca^{2+}]_i$ is detectable in a subset of cells. Interestingly, in the majority of cells the Ca^{2+} signal induced by NO is enhanced by pretreament with 100 pM progesterone (10 min), confirming that this is sufficient to modify the Ca^{2+} signalling apparatus of the cell (128), Thus it is possible that sperm do show physiological responses involving $[Ca^{2+}]_i$ signalling upon exposure to pM doses of progesterone.

Secondly, are receptor-mediated mechanisms of Ca^{2+} mobilisation induced by nM-microM doses of

progesterone, of which we have relatively detailed knowledge (if not understanding), relevant to physiological responses that occur at pM 'chemotactic' doses? Though the nature of the receptor(s) for progesterone is still far from clear, dose-dependence and binding studies have suggested that two binding sites may contribute to the known, Ca^{2+}-dependent, effects of progesterone on mammalian sperm (123; see above) However, picomolar doses of progesterone will not act at the low affinity (micromolar) steroid receptor on human sperm (115) and even the reported higher affinity progesterone-specific receptor (which has a Kd of 600 pM by displacement assay [115]) should be only weakly activated by the 10 pM dose that shows the strongest chemotactic activity (106). Thus if Ca^{2+} signalling is involved in the chemotactic activity of progesterone, it may well require that we re-examine the cellular response, using high sensitivity imaging techniques, in order to begin to understand the processes involved. This should be a high priority.

6. PERSPECTIVE

The last 5 years have seen considerable progress in our understanding of Ca^{2+} signalling in sperm and particularly its contribution to the regulation of motility and chemotactic behaviour. However, it is fair to say that different models or 'stories' have emerged from the activities and interests of different laboratories. The central role of CatSper in hyperactivation is beyond dispute and it is possible to generate a coherent model for hyperactivation involving regulation of the channel by pH (and pH-regulated hyperpolarisation of membrane potential) leading to Ca^{2+} influx, tonic elevation of $[Ca^{2+}]_i$ and hyperactivation. Similarly, mobilisation of stored Ca^{2+} in the neck region of rodent and bovine sperm clearly promotes hyperactivation, at least for a period. But how do these two processes interact? - are they employed simultaneously or separately in response to different stimuli? Xia *et al*, (90) observed that activation of CatSpers in the principal piece of mouse sperm results in a $[Ca^{2+}]_i$ signal that propagates to the sperm head. A contribution of calcium-induced calcium release in the neck region to this process appears likely, but can this interaction work in the opposite direction, mobilisation of stored Ca^{2+} leading to activation of CatSpers? Ca^{2+} influx downstream of olfactory receptor activation is believed to be mediated by generation of cAMP and consequent gating of Ca^{2+}-permeable membrane channels (129). The $[Ca^{2+}]_i$ signal generated by this process leads to chemotactic turns and orientation of cells up a chemotactic gradient. This $[Ca^{2+}]_i$ signal occurs in the midpiece and flagellum (129) and yet it's effect is apparently quite separate and discrete from those that induce hyperactivation. How is this achieved? Potential mechanisms include the ability of the Ca^{2+}-regulated elements in the flagellum to differentiate between signals on the basis of their temporal characteristics and also the possibility of selective regulation of the Ca^{2+}-sensitivity of target proteins by other signalling cascades? Is asymmetry in hyperactivation and during a chemotactic turn achieved differently? The recent suggestion by Xia *et al*, (90) that Ca^{2+} mobilisation in the midpiece regulates mitochondrial activity, providing an increase in ATP-generation during hyperactivation, provides yet more complexity and subtlety to the story.

The activities carried out by sperm in traversing the female tract, finding the cumulus-oocyte complex and undergoing key processes such as hyperactivation and acrosome reaction at the appropriate time and place are, in such a simple cell, amazingly complex (1). Compared to ten years ago, we now have a wealth of data relating the various types of spatiotemporal signal that occur in sperm to the activities that they regulate. The simplicity of the sperm's structure is such that the development of a model for sperm Ca^{2+} signalling (quite possibly with significant differences between the sperm of different organisms), which incorporates our observations and predicts the sperm's ability to select and regulate its behaviour is a realisable goal.

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